ON THE DEVELOPMENT AND PREVENTION OF PERIODONTAL DISEASE IN THE BEAGLE DOG

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PREFACE

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Lindhe, J., Hamp, S.-E., Löe, H. & Rindom Schiött, C. 1970. Influence of topical application of chlorhexidine on chronic gingivitis and gingival wound healing in the dog. Scand J Dent Res 78: 471-478.

II. Hamp, S.-E., Lindhe, J. & Heyden, G. 1972. Experimental gingivitis in the dog. An enzyme histochemical study. Arch Oral Biol 17: 329-337.

III. Lindhe, J., Hamp, S.-E. & Löe, H. 1973. Experimental periodontitis in the Beagle dog. J Periodont Res 8: 1-10.

IV. Hamp, S.-E., Lindhe, J. & Löe, H. 1973. Long term effect of chlorhexidine on developing gingivitis in the Beagle dog. J Periodont Res 8: 63-70.

V. Hamp, S.-E. & Emilson, C.G. 1973. Some effects of chlorhexidine on the plaque flora of the Beagle dog. J Periodont Res Suppl 12: 28-35.

INTRODUCTION

Epidemiological studies, clinical trials and animal experiments have revealed a close relationship between microbial dental plaque and gingival inflammation (for reviews, see Socransky 1970, Waerhaug 1971, Genco 1973). A basis for studying the gingiva in health and in disease was established when Löe, Theilade & Jensen (1965), Theilade et al. (1966), Egelberg (1964, 1967), Oliver, Holm-Pedersen & Löe (1969) and Attström (1971) demonstrated in humans and dogs that (i) a clinically healthy state of the gingiva could be achieved in areas kept free from dental plaque by mechanical cleaning of the teeth and (ii) a healthy gingiva could be rendered inflammatory simply by allowing microorganisms to colonise on the gingival third of tooth surfaces.

In a longitudinal study in the dog Saxe et al. (1967) produced some evidence for a relationship not only between the development of plaque and gingivitis, but also between prolonged accumulation of plaque and calculus and the initial phases of periodontal breakdown. On the basis of these reports and earlier observations in humans by e.g. Weinmann (1941), Goldman (1945), Fish (1948) it has been assumed that periodontal disease starts as an inflammation of the gingiva and gradually involves increasingly deeper parts of the supraalveolar connective tissue and finally results in (1) loss of collagen fibres below the cemento-enamel junction, (2) an apical shift of the epithelial attachment, and (3) bone resorption when the inflammatory cell infiltrate reaches the alveolar bone.

Mechanisms of non-inflammatory nature have also been discussed in relation to periodontal tissue destruction e.g. degenerative periodontosis (Orban & Weinmann 1942, Goldman 1947), passive tooth eruption (Gottlieb & Orban 1933, Gargiulo, Wentz & Orban 1961), the bone factor concept (for review, see Glickman 1972) including a variety of systemic influences upon the alveolar bone response to extrinsic and intrinsic stimuli.

The first aim of the present studies was to analyse in detail some

characteristics of the early gingival lesion and, if possible, the successive stages of the development of a periodontal lesion in an originally healthy periodontium.

A plaque-free dentition is of importance not only for the maintenance of gingival health, but also for the prevention of tooth decay (Löe, von der Fehr & Rindom Schiött 1972, Lindhe & Axelsson 1973). Therefore, a number of plaque-control programs have been designed and tested (for reviews, see Kock 1967, Lindhe, Lundgren & Nyman 1970, Bay 1972). As, however, the maintenance of a plaque-free dentition in humans by mechanical means is obviously arduous, attempts have been made to utilise various chemicals to substitute or supplement mechanical cleaning of the teeth (for review, see Gjermo 1972).

The most promising, so far, is the introduction into an intra-oral milieu, of an earlier widely used antimicrobial agent with excellent bactericidal properties and very low toxicity (for review, see Longworth 1971), namely chlorhexidine digluconate (Hibitane®, ICI, U.K.). Thus, Löe (1969) and Löe & Rindom Schiött (1970a,b) found in humans that when mouth-rinsing with chlorhexidine substituted mechanical cleaning of teeth the formation of dental plaque was completely inhibited, but also that experimentally induced mild gingivitis disappeared. Furthermore, it was demonstrated that caries did not develop, even with frequent additions of sucrose, as long as the mouth was also rinsed with chlorhexidine (Löe et al. 1972). Since 1969 a number of short-term experiments in humans and animals have supported the plaque-inhibitory effect of chlorhexidine (for review, see Hirst 1972). This, therefore, created the need for long-term studies on the effect of chlorhexidine on plaque, calculus and gingivitis.

The second aim of this series of investigations was to assess the prevention of periodontal disease, if possible, by plaque-control programs comprising mechanical cleaning of the teeth or topical application of chlorhexidine digluconate.

MATERIAL AND METHODS

Animals

The experiments were performed on dogs (beagles). Animals belonging to different groups (experimental and control) were always kept separated from each other. Prior to the experiments the dogs were inoculated against distemper and canine hepatitis. Veterinary examination and chemical analysis of the blood were performed as regular intervals during the experiments.

Diet

The dogs were subjected to a hard-diet regimen during preparatory periods; either raw whole bovine tracheas with additional tablets of vitamins, calcium phosphate and ferrous sulphate (Egelberg 1965) (II, III, IV) or dog pellets (Pris®, Astra, Sweden) (V). During the experimental periods the animals were given a soft diet consisting of minced tracheas with supplementary vitamins and minerals (I); dog sausages, potato flour, sugar and water (II); or dog pellets and soya meal mixed in water (III, IV, V).

Gingival health

In order to achieve gingival health the animals were subjected to carefully controlled oral hygienic measures during the preparatory periods: 1) mechanical tooth cleaning with rubber cups and pumice three times a week for 5 weeks (II), or 2) mechanical tooth cleaning according to Bass (1948) twice a day with toothbrush and dentifrice for 8 weeks (III, IV, V). In the study presented in paper I gingival health was achieved within 6 weeks by the use of 2 % chlorhexidine digluconate applied topically once a day to the teeth and gingiva.

Clinical examinations

In the study reported in paper I all teeth except the incisors, second and third molars were utilised for the clinical examinations. In later studies (III and IV) reference points (amalgam fillings) were made on the buccal surfaces of selected teeth (P^4, P^3, P^2, C, I^3 and M_1, P_4, P_3, P_2 and C) and all clinical measurements except sampling of crevicular leukocytes were confined to these areas.

The degree of plaque and calculus formation was assessed according to the criteria of the Plaque Index (Silness & Löe 1964) and the Debris and Calculus Index systems (Greene & Vermillion 1960). The gingival conditions were estimated by (1) using the Gingival Index system (Löe & Silness 1963), (2) measuring the degree of gingival exudation, and (3) measuring the number of crevicular leukocytes.

The Plaque Index system is built up of four different scores. The following criteria were used: 0 = no plaque; 1 = a film of plaque adhering to the gingival third of the tooth. The plaque may be seen in situ only after application of a disclosing solution or by examination of the tooth surfaces with a probe; 2 = moderate accumulation of soft deposits within the gingival third of the tooth. The deposits can be seen with the naked eye; 3 = abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

The scores of the Debris Index system were used according to the following criteria: 0 = no debris or stain present; 1 = soft debris covering not more than one third of the tooth surface, or the presence of extrinsic stains without other debris regardless of surface area; 2 = soft debris covering more than one third, but not more than two thirds of the exposed tooth surface; 3 = soft debris covering more than two thirds of the exposed tooth surface.

The scores and criteria of the Calculus Index used were: 0 = no calculus present; 1 = supragingival calculus covering not more than one third of the exposed tooth surface; 2 = supragingival calculus covering more than one third, but not more than two thirds, of the exposed tooth surface and/or the presence of discrete areas of subgingival calculus around the cervical portion of the tooth; 3 = supragingival calculus covering more than two thirds of the exposed tooth surface and/or a continuous band of subgingival calculus around the cervical portion of the tooth.

The scores and criteria of the Gingival Index system are: 0 = absence of inflammation; 1 = mild inflammation - slight change in colour and little change in texture; 2 = moderate inflammation - moderate glazing, redness, oedema, and hypertrophy. Bleeding on pressure; 3 = severe inflammation - marked redness and hypertrophy. Tendency to spontaneous bleeding. Ulceration.

Gingival exudate was sampled according to the intra-crevicular technique described by Löe & Holm-Pedersen (1965) on filter paper strips 1.5 x 15 mm cut from Munktell filter paper No. 3 (I, II) or Whatman Chromatography paper No. 4 (III, IV). After standardised drying of the area with a blast of air the strips were placed very gently at the orifice of the crevices. The sampling period was always 3 minutes, after which the strips were dried and stained in a 0.2 % alcoholic solution of ninhydrin. The length of the stained areas was measured to the nearest of 0.2 mm with a calibrated magnifying glass.

Crevicular leukocytes were sampled according to the intra-crevicular technique described by Attström & Egelberg (1971). Styroflex® films (Norddeutsche Seekabelwerke, W. Germany) 0.05 x 1.5 x 15 mm, were gently inserted as deeply as possible into the crevices and then immediately removed. After the films had been mounted and stained with May Grünwald-Giemsa the cells were counted in a microscope. Two methods for quantitation were used. In paper I the density of inflammatory cells on the films was assessed according to the following criteria: - = 0 cells; + = $<$ 25 cells; ++ = 25 - 400 cells; +++ = $>$ 400 cells. In paper III and IV the following criteria were used: 1 = scattered cells; 2 = $<$ 100 cells; 3 - 5 = cells covering the entire stained surface: 3 = slight density, 4 = moderate density, 5 = marked density of leukocytes.

The depth of the gingival crevices and the distance from the apical border of the reference point to the bottom of the crevice were determined of the nearest 0.5 mm with a specially designed, graduated pocket probe (III, IV). The measurements correspond to the clinical pocket depth (distance between the gingival margin and the bottom of the clinical crevice) and the clinical loss of attachment (distance between the amalgam filling and the bottom of the clinical pocket minus the corresponding distance on day zero, i.e. at the beginning of the experiment).

Standardised roentgenograms of the marginal alveolar bone (III, IV) were produced in accordance with a method described by Eggen (1969). Roentgenograms of M_1, P_4 and P_3 were obtained on day zero and again every sixth month. The X-ray examination was designed to demonstrate qualitative rather than quantitative changes.

During the experiments colour photographs were taken regularly with Kodak Ektachrome-X® films and a Medical-Nikkor® camera.

Biopsy procedures

Soft tissue specimens (I, II) from the buccal gingiva were obtained with a standardised technique originally described by Holm-Pedersen & Löe (1967). With a double-bladed Bard Parker scalpel and a fixed distance of 3 mm between the blades two vertical incisions were made to the crest of the alveolar bone. Following a horizontal incision the tissue section was carefully dissected free, removed, immediately placed in ice-cold Hank's balanced salt solution and cut vertically into two equal pieces. One half was selected for subsequent enzyme histochemical analyses and therefore mounted on cryostat chucks and immediately frozen in liquid nitrogen. The tissue specimens were stored at -80°C.

The tissue specimens obtained for histological-morphological analysis (II) were fixed in 1.7 % glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4. They were then dehydrated in graded ethanols and finally embedded in Epon. Sections of 1 µm were obtained in a LKB ultramicrotome and stained with toluidine blue.

Soft and hard tissue specimens (III, IV) were obtained from buccal gingiva with the technique described by Schroeder et al. (1973b). The clinical crown was eliminated with high speed dental equipment. The pulp channels were utilised to cut a deep cavity, the bottom of which always was located 2-3 mm apical to the marginal bone crest. The cavity was then widened in buccal direction, leaving only a narrow enamel-dentine wall between the buccal marginal tissue and the cavity in the tooth. From the mesial and distal ends of the cavity, vertical clefts were cut in buccal direction through the tooth, alveolar bone and gingiva. A horizontal cut, at the level of the cavity bottom, excised the marginal tissue portion.

The specimens were transferred to a fixative containing 5 % glutaraldehyde and 4 % paraformaldehyde buffered to pH 7.3 with 0.02 M sodium cacodylate (Karnovsky 1965). Twentyfour hours later the tissues were decalcified for 3 months in 0.1 M EDTA combined with

4 % glutaraldehyde and 0.2 M sucrose (Warshawsky & Moore 1965). After postfixation in 1.33 % OsO_4 and 0.067 M s-collidine buffer the tissue sections were dehydrated in graded ethanols, embedded in Epon and sections of 1 µm were made. Staining was performed with a PAS and toluidine blue technique described by Schroeder & Münzel-Pedrazzoli (1973).

Histochemical registration of enzyme activities

Serial sections were cut at -20°C in a Kryostat 64 (System Dittes-Duspiva, Heidelberg, Germany). Activities of lactate dehydrogenase (EC 1.1.1.27) and malate dehydrogenase (EC 1.1.1.37) in the tissue sections were studied according to a modification of the Barka & Anderson (1963) procedure, described by Heyden & From (1969). The sections were incubated for 30 minutes at 37°C in 0.5 ml microcells (Jones 1964). ATP phosphohydrolase (ATPase, EC 3.6.1.4) activity was demonstrated by a modification of the lead method (Wachstein & Meisel 1957) at pH 7.2, according to Jacobsen & Leth Jörgensen (1969). Controls for interfering non-enzymic staining reactions were made after enzyme inhibitory treatments (10 % formalin on tissue sections) and incubation of the sections in media without enzyme substrates. Staining with haematoxylin and eosin and the van Gieson technique was used for histological references of the distribution of the histochemical reactions.

Bacteriological analysis

Dental plaque (V) was sampled according to a technique described by Jordan, Krasse & Möller (1968). The sample was then placed in VMG II medium (Möller 1966) for transportation.

Following dilution 1:10 in a 3 % trypticase solution (BBL) 0.1 ml of the solution was spread on blood agar plates. Subsequently, filter paper discs containing an increasing amount of chlorhexidine digluconate (5, 10, 50, 200 and 500 µg) were placed on the surface of the plates. After prediffusion at room temperature for 30 minutes, the agar plates were incubated in a jar in an ambient atmosphere of 95 % N_2 and 5 % CO_2 for 24 hours at 37°C. The areas of growth inhibition

were calculated, and each value was plotted against the logarithm of the amount of chlorhexidine in the discs, according to a method described by Linton (1958) and Emilson & Krasse (1972), originally designed by Ericsson, Högman & Wickman (1954) in antibiotic assays.

The chlorhexidine-sensitivity of the plaque flora was also studied with a dilution-plate method on trypticase soy agar plates containing 0.2 % glucose and 0, 20, 80 and 320 µg/ml chlorhexidine digluconate. The plates were incubated aerobically for 24 hours at 37°C. One of the predominating colonies was selected from each plate with the highest concentration that did not completely inhibit growth, and the isolated strains were identified[x] according to Cowan & Steele (1965) and Edwards & Ewing (1972).

Statistical methods

Student's t-test was used in study V. In the experiments presented in papers III and IV the data were transferred to data cards and analysed statistically[xx] with the aid of an IBM 3/65 computor. The analysis of changes of different variables was based upon individual mean values y_{ij}. Such a value y_{ij} [a score from dog number (i) during the observation period (j) after the beginning of the experiment] represents the mean value of measurements from a number of predetermined measuring points, either from molars, premolars, incisors or from all the predetermined measuring points. For every y_{ij} the following model of a block design was used:

$$y_{ij} = \mu + D_i + T_j + \varepsilon_{ij}$$

By analysis of variance it is possible to estimate the residual error, i.e. the variance of the random component ε_{ij}. On the basis of such an estimate the so-called critical difference ($\overline{d}_c$) is calculated according to Tukey's method of comparing all possible pairs of means. The difference between any two time mean values is hence significant at the 5 per cent level if it is greater than $\overline{d}_c$. The critical differences were used to detect significant changes in the time course of the different periodontal parameters (Cf. Li 1964).

x) This part of the study was performed with the kind help of Prof. Jan Carlsson, Department of Oral Microbiology, University of Umeå.

xx) This part of the study was performed with the kind help of Prof. Esbjörn Carlström, University of Göteborg.

RESULTS

I. INFLUENCE OF TOPICAL APPLICATION OF CHLORHEXIDINE ON CHRONIC GINGIVITIS AND GINGIVAL WOUND HEALING IN THE DOG

The aim was to study the effect of repeated topical application of chlorhexidine on chronically inflamed gingiva and standardised gingival wounds.

Material and methods. For 5 months before the experiment proper 5 dogs (beagles) were fed a soft diet. By the beginning of the experiment they had well established gingivitis around all the teeth. In 3 dogs a 2 % aqueous solution of chlorhexidine digluconate was applied once a day to the teeth and gingiva of the left half of the jaws for 42 days. Corresponding teeth and gingiva of the right halves were treated with saline. Gingival specimens were obtained at the beginning and at the end of the experiment.

In the remaining 2 dogs standardised gingivectomies were made of the buccal gingiva of 6 teeth. The gingival wounds (produced by the surgical procedure) in one dog were then treated once a day with 2 % chlorhexidine digluconate; those in the other dog with saline. Gingival specimens for histological examination were obtained at the beginning and at the end of the experiment.

Plaque formation, gingivitis, gingival exudate and the number of migrating crevicular leukocytes were assessed in both experiments.

Results. At the end of the study teeth treated with chlorhexidine digluconate were completely devoid of plaque, the gingivae appeared healthy and the scores of gingival exudation and crevicular leukocytes were low. Random gingival specimens obtained at the beginning of the experiment consistently showed pronounced infiltration of inflammatory cells subjacent to the crevicular epithelium. The gingiva of tissue specimens obtained at the end of the study was practically devoid of inflammatory cells.

During the wound healing period the inflammatory reactions were less pronounced in the dog treated with chlorhexidine digluconate than in the control treated with saline. Thus, after surgery gingival exudation from all excised areas in both the experimental and the control dog reached a maximum on day 4. Then exudate from the chlorhexidine-treated areas decreased to almost zero by day 42. In the control, however, the exudate scores did not change substantially during the period of observation. The tissue specimens obtained at the beginning of the experiments showed pronounced infiltration of inflammatory cells in the connective tissue subjacent to the crevicular epithelium. At the end of the experiments the gingival areas treated with chlorhexidine digluconate were practically devoid of inflammatory infiltrates, whereas tissue specimens from saline-treated gingiva exhibited large areas of round cell infiltration.

Conclusion. Well established gingivitis disappeared within 6 weeks' daily topical application of chlorhexidine digluconate to the teeth and gingiva. During the same period gingival wounds treated with chlorhexidine digluconate healed with only minor persistent signs of inflammation.

II. EXPERIMENTAL GINGIVITIS IN THE DOG. AN ENZYME HISTOCHEMICAL STUDY

The aim was to assess possible alterations in cell metabolism in the gingival epithelia in association with the initial development of inflammation in previously healthy gingiva.

Material and methods. During a preparatory period of 5 weeks gingival health was achieved in 5 dogs (beagles) by repeated mechanical tooth cleaning. Accumulation of dental plaque was afterwards stimulated by feeding the animals a soft and sticky diet. The state of the gingiva was assessed from the degree of gingival exudation and from examination of gingiva specimens.

Results. Healthy gingiva, characterised by the absence of gingival exudation as well as of inflammatory cell infiltrates in the connective tissue, displayed the following enzyme histochemical pattern. The intracellular localisation and distribution of lactate and

malate dehydrogenase activities were found within the stratum basale and the deeper portions of the stratum spinosum. In the basal cells and in the deeper parts of stratum spinosum enzyme activity was usually found in the cytoplasm between the nucleus and the basement membrane. Within the middle and superficial parts of stratum spinosum, the enzyme activity had a perinuclear distribution. The activity showed a decreasing gradient towards the surface cell layers of the epithelium, which were completely devoid of visible enzyme activity reactions.

In the crevicular epithelium, the enzyme activity in the cytoplasm was found perinuclearly except in the basal cells, where the activity was most concentrated in the parts of the cytoplasm bordering the basement membrane. The outher zone of degenerating cells was devoid of enzyme activity.

ATPase activity was observed in the middle part of stratum spinosum of the oral epithelium and was confined mainly to the region of the plasma membrane. The hydrolytic enzyme activity decreased towards the granular and basal layers. No ATPase activity was found in the crevicular epithelium, whereas high activity was demonstrated in the endothelial cells of the crevicular vessels.

During the state of subclinical gingivitis, characterised by a clinically healthy appearance but with the presence of minor gingival exudation (<0.3 mm on filter paper strips) and accumulations of inflammatory cells in the connective tissue close to the marginal fifth of the crevicular epithelium, the gingivae showed the following enzyme histochemical pattern. Lactate and malate dehydrogenase activities were present within the oral epithelium (at the level of the inflammatory cell infiltrate) in all but the superficial cell layers. In comparison with healthy gingiva, the activities had increased to such an extent from the stratum basale towards the stratum granulosum that differences in intensity between the different cell layers seemed to be almost negligible.

In the crevicular epithelium, at the level of the inflammatory cell infiltrate, lactate and malate dehydrogenase activities were present in all cell layers except in the outer zone of degenerating cells. Intense lactate and malate dehydrogenase activities were

noted also in the endothelial cells of the crevicular vessels, especially in vessels surrounded by inflammatory cells.

Sections stained for ATPase activity showed, as in healthy gingiva, high activity within the middle part of stratum spinosum of the oral epithelium. Hydrolytic enzyme activity was also demonstrated in the basal part of stratum spinosum of the oral epithelium and in the basal cell layer of the crevicular epithelium. An intense ATPase activity was found in the endothelial cells of the crevicular vessels, especially in vessels surrounded by inflammatory cells.

Conclusion. In early gingival inflammation the histochemical changes comprise enhanced ATPase and lactate and malate dehydrogenase activities in oral and crevicular epithelial cells close to inflammatory cell infiltrates and in endothelial cells of vessels within the inflamed areas.

III. EXPERIMENTAL PERIODONTITIS IN THE BEAGLE DOG

The aim was to study the sequential stages in the development of a periodontal lesion in a previously healthy periodontium.

Material and methods. The material consisted of 20 female 10 month old dogs (beagles) divided into 2 equal groups. During a preparatory period of 8 weeks the dogs were subjected to meticulous oral hygienic procedures. This resulted in excellent gingival health as evaluated by clinical inspection and gingival exudate measurements.

Throughout the experiment all dogs were given a diet favouring gross accumulation of plaque. From day zero the teeth in the left halves of the jaws of the control dogs, but not in the experimental dogs, were carefully cleaned twice a day with a toothbrush and dentifrice.

Results. Plaque soon formed on the uncleaned teeth in the experimental dogs, in which the molars and posterior premolars showed large accumulations of plaque already within 1-2 weeks. After approximately 3 weeks mineralised deposits were observed on the buccal surfaces of maxillary molars and posterior premolars. Six months from the beginning of the experiment these deposits were no longer

located only supragingivally, but often also subgingivally. In the anterior premolars subgingival calculus was first seen at the 12 month examination. The incisors had no visible plaque before the 18 month examination.

A mean Gingival Index ~ 1 indicating slight redness and oedema (mild gingivitis) was scored by molars after one month, by premolars after 4-6 months, and by incisors after 12 months. Moderate to severe gingivitis was not demonstrable until after 4 months around molars, 12 months around premolars, and 18 months around incisors. Subclinical signs of gingivitis, characterised by gingival exudation and enhanced migration of crevicular leukocytes, were, however, registered around molars already during the first week after the tooth cleanings had been terminated.

The first significant increase in clinical pocket depth occurred in molar and premolar areas at the 2 month examination, and in incisor areas 6 months later. The first significant clinical loss of fibre attachment was noted after the molars had not been cleaned for 6 months. The corresponding period for premolars and incisors was 8 months. From the sixth month onwards a slight recession of the gingival margin of upper molars and posterior premolars was observed. After 18 months, roentgenograms showing reduced density of bone were obtained from interproximal areas of P_4 and M_1.

Dental plaque was observed only occasionally in the control dogs. It showed no consistent pattern of distribution. No significant increase in Gingival Index, gingival exudate scores, number of crevicular leukocytes, clinical pocket depth, clinical loss of attachment, gingival recession or change in bone level could be recognised during the experimental period.

Conclusion. Complete neglect of oral hygiene results in plaque and calculus formation, and, as a consequence, in the development of gingival and periodontal lesions. Three stages may be recognised:

1) Subclinical gingivitis, characterised by rapidly increasing gingival exudation and enhanced migration of crevicular leukocytes (i.e. signs of acute inflammation).

2) Clinical gingivitis, characterised by changes in gingival colour, texture and bleeding tendency, but only minor further alterations in the number of crevicular leukocytes, as compared to the subclinical state.
3) Periodontal breakdown, characterised by loss of the subgingival fibre attachment in areas of clinical gingivitis.

The present study also showed that meticulous daily mechanical cleaning of the teeth kept the periodontal tissues clinically and histologically free from inflammation.

IV. LONG-TERM EFFECT OF CHLORHEXIDINE ON DEVELOPING GINGIVITIS IN THE BEAGLE DOG

The aim was to study the long-term effect of chlorhexidine on the development of plaque, calculus and gingivitis in the dog.

Material and methods. 20 dogs (beagles) were divided into 2 groups of 10, an experimental group and a control group, and given a soft plaque-forming diet for 12 months. In the experimental group a 0.2 % aqueous solution of chlorhexidine digluconate was applied topically twice a day to the teeth and gingiva of the right half of the jaws and allowed to act for 2 minutes. The controls were treated in the same way, but with saline. Plaque, calculus and gingivitis were assessed after 7, 14, 21, 28 days and after 2, 4, 6, 8, 12 months.

Results. Topical application of 0.2 % chlorhexidine digluconate prevented visible dental plaque formation for 4 months. Between the 4 and 6 month recordings plaque accumulated in minor amounts on molars and posterior premolars. During the final 6 months plaque continued to develop on the tooth surfaces.

Simultaneously with plaque formation isolated flecks of light yellow-brownish mineralised deposits resembling ordinary calculus were recognised on the surfaces of posterior teeth. These deposits were easily distinguished from discoloured deposits observed within the first 2 weeks of chlorhexidine treatment. Throughout the experiment the stained deposits gradually turned deep brown to black and increased in thickness and extent.

In chlorhexidine-treated areas the Gingival Index and gingival exudation showed only minor alterations during the first 6 months. Thereafter the scores increased, especially in molars and posterior premolars.

In the saline-treated animals dental plaque was demonstrated in all tooth regions within the first month. Mineralised deposits were seen on molars after the teeth had not been cleaned for 2-3 weeks, and accumulated successively throughout the experimental period. Gingivitis developed during the first month and became gradually more and more pronounced in all tooth regions.

Conclusion. Topical application of 0.2 % chlorhexidine digluconate twice a day to teeth and gingiva in dogs prevents for almost 6 months the development of clinical signs of plaque and gingivitis. Once initiated, however, plaque and gingivitis increasingly develop also in chlorhexidine-treated areas.

V. SOME EFFECTS OF CHLORHEXIDINE ON THE PLAQUE FLORA IN THE BEAGLE DOG

The aim was to study the sensitivity, to chlorhexidine, of dental plaque microorganisms obtained from dogs (beagles) after prolonged treatment with the compound.

Material and methods. Three series of experiments were performed during which the animals were given a diet favouring gross plaque formation. In Series I, the teeth of 10 dogs were sprayed twice a day with 0.2 % chlorhexidine digluconate and the teeth of 10 controls with saline for 6 months. In Series II, the same treatment was given for 18 months to 16 dogs (8 experimental dogs, 8 controls). In Series III (three dogs) the teeth of the left half of the jaws were treated with two concentrations of chlorhexidine digluconate for 5 + 5 weeks (2 and 0.1 %, respectively), while the teeth of the right half of the jaws were treated with saline. During a post-experimental period of 3 months all teeth were sprayed with saline.

Dental plaque was sampled at the end of the experimental periods;

in Series III also after 1, 3, 6 and 13 weeks of the post-experimental period. A modification of "the paper disc method" (Ericsson, Högman & Wickman 1954) was used as a primary sensitivity test to evaluate the sensitivity of the plaque bacteria to chlorhexidine digluconate. The samples obtained from dogs after an experimental period of 18 months (Series II) were examined also with a dilution-plate method on trypticase soy agar. One of the predominant colonies from each culture plate was selected and the isolated strains were identified.

Results. The sensitivity of plaque microorganisms to chlorhexidine was significantly reduced in samples from dogs treated with 0.2 % chlorhexidine digluconate twice daily for 6 and 18 months, compared with plaque bacteria sampled from saline-treated control dogs. In samples from the experimental dogs a significant portion of the microflora grew on agar plates containing 80 µg/ml chlorhexidine digluconate and in all dogs except one, also on plates containing 320 µg/ml. On the other hand, in samples from the controls, only a minor portion of the flora grew at 20 µg/ml. The isolated strains with reduced sensitivity to chlorhexidine were gram-negative rods; Proteus, Klebsiella and Citrobacter were isolated from the chlorhexidine group, while Flavobacterium, Acinetobacter, Escherichia and Klebsiella were found in samples from the saline-treated dogs.

In the experiments in Series III neither plaque nor gingivitis could be observed in the chlorhexidine-treated dogs during the first 5-week period. At the end of the second 5-week period some plaque was demonstrated in the posterior teeth of both groups. The chlorhexidine-sensitivity of microorganisms from chlorhexidine-treated teeth was less than that of plaque bacteria from saline-treated teeth. During the post-experimental period the sensitivity of plaque bacteria from chlorhexidine-treated teeth slowly approached the corresponding values found for saline-treated teeth.

Conclusion. Prolonged use of chlorhexidine digluconate in the oral cavity of the dog results in a dental plaque flora with reduced sensitivity to the substance.

DISCUSSION

The present long-term experiments (18 months) demonstrated that it is possible to achieve and maintain a normal appearance of the gingiva by preventing microbial colonisation in the dento-gingival region in the dog. The experiments also showed that (i) gingivitis can be induced by allowing microbial colonisation in the dento-gingival region; (ii) gingival inflammation is initially characterised by flow of gingival fluid, increased migration of crevicular leukocytes and enhanced lactate and malate dehydrogenase and ATPase activities in oral and crevicular epithelial cells close to inflammatory cell infiltrates and in endothelial cells of vessels within the inflamed areas; (iii) the gingiva shows no change in colour or texture until gross plaque has formed for 3-4 weeks, and (iiii) with prolonged plaque accumulation gingival inflammation gradually develops into periodontitis characterised by an apical spread of the leukocyte infiltrate, an apical shift of the crevice epithelium by loss of fibre attachment, and alveolar bone resorption.

Furthermore, it was demonstrated that the replacement of mechanical tooth cleaning with a chemical antibacterial agent, chlorhexidine digluconate, inhibits the development of supragingival plaque and gingivitis for almost 6 months. Once started, plaque gradually develops on the gingival third of tooth surfaces, and later gingivitis appears. Plaque development in chlorhexidine-treated tooth areas was shown to be the result of colonisation of bacteria with less sensitivity to the drug.

Healthy gingiva

In the dog (beagle) mechanical cleaning of the teeth plus scaling and polishing once a week for 5-8 weeks resulted in a clinically healthy appearance of the gingiva, i.e. pale pink colour, firm consistency and no bleeding on gentle probing. No flow of gingival exudate could be registered on filter paper strips placed at the entrance of the gingival crevice and only minor amount of crevicular

leukocytes could be demonstrated on Styroflex films inserted into the gingival crevice. Gingiva specimens with these clinical characteristics consistently displayed a non-inflammatory appearance, i.e. did not reveal round cell infiltrations, though scattered leukocytes regularly migrated into the crevicular epithelium.

The observation that regular cleaning of the teeth resulted in the disappearance of all signs of inflammation of the gingiva is in agreement with the finding by Oliver et al. (1969) that human biopsies of clinically healthy gingiva contained, at most, only few inflammatory cells beneath an intact crevicular epithelium. In the dog, Attström (1970) found that 10 specimens out of 21 from clinically healthy buccal gingiva could be classified as histologically healthy, i.e. complete absence of round cell infiltrates. On the other hand, in a comparative histological and clinical study in man Zachrisson & Schultz-Haudt (1968) found that clinically healthy gingiva always showed some inflammatory cells in the connective tissue "near the pocket epithelium". The discrepancy between the present findings and those reported by Zachrisson & Schultz-Haudt (1968) can probably be ascribed to differences in the clinical criteria used for classification of a gingiva as healthy.

Determinations of the crevicular leukocytes showed that a gingiva fulfilling the clinical and histological criteria of health contained leukocytes in the gingival sulcus and crevicular (junctional) epithelium. The majority of these so-called crevicular leukocytes (Attström 1970) found on Styroflex films can be classified as neutrophils. This finding corroborates data presented by Egelberg (1963), Attström (1970), Schroeder (1970), Schroeder & Listgarten (1971). Recently, Helldén et al. (1973) showed that material sampled from clinically healthy gingival crevices contained substances, which in experiments performed in vitro were chemotactic for neutrophils. Crevicular material from healthy gingiva contains only few bacteria, but several leukocytes and desquamated epithelial cells. Helldén et al. (1973) therefore suggested that chemotactic substances operating in healthy crevices are derived mainly from non-bacterial sources. It is well known that neutrophils release substances which are chemotactic for other neutrophils (e.g. Hurley & Spector 1961, Golub & Spitznagel 1966, Borel, Keller & Sorkin 1969). In a recent study Tynelius-Bratthall & Lindhe (1973) found

that rabbit neutrophil lysosomes supplied neutrophil chemotactic activity *in vitro*.

Subclinical gingivitis

When oral hygiene was completely neglected in dogs fed a soft diet microbial plaques accumulated on the teeth. These plaques were at first confined only to the gingival third of the tooth surfaces. Within the first week after the initiation of plaque formation most gingival units exhibited flow of exudate and enhanced migration of crevicular leukocytes (III). This means that since definite alterations in colour and texture of the gingiva are not seen until after 3-4 weeks of undisturbed plaque accumulation, gingival exudation and an increased number of crevicular leukocytes must be regarded as early subclinical signs of gingivitis.

Gingival tissue sections obtained during the early period of subclinical gingivitis consistently contained small areas of infiltrated connective tissue (II). The accumulations of inflammatory cells were always found immediately beneath the marginal fifth of the crevicular epithelium. The epithelium close to connective tissue areas with inflammatory cell infiltrates exhibited a raised activity of lactate and malate dehydrogenase. Alterations in the enzyme activities of the epithelial cells were observed simultaneously with an increase in enzyme activity within the local endothelial cells, which may reflect an altered vascular function, although this was not particularly studied in these experiments. Results showing an altered microvascular topography and function in inflamed dento-gingival tissues were reported by e.g. Egelberg (1967) and Hansson, Lindhe & Brånemark (1968). The observation was made in paper II that the distribution of the enzyme activities was identical in completely healthy specimens and in non-infiltrated areas of subclinically inflamed specimens. This finding indicated that early pathological alterations within the gingiva are confined to the zone of inflammatory cell infiltrates.

The early onset of gingival exudation indicates alterations of the vessels within the crevicular plexus. In experiments by e.g. Egelberg (1967), Hansson et al. (1968), Hock & Nuki (1971) crevicular

vessels close to an accumulating dental plaque were shown to suffer from microvascular dysfunctions, become labelled with colloidal carbon and exhibit leakage of plasma proteins. Söderholm & Egelberg (1973) recently reported that after only a few days of dental plaque accumulation morphological alterations of the marginal vascular bed developed. The initial alteration was a widening of the venular aspect of the very marginal part of the crevicular plexus.

The results reported in paper III showed that there was a relationship not only between plaque formation and gingival exudation, but also between the accumulation of plaque and an increased number of crevicular leukocytes. The increased leukocyte transmigration is most likely explained by the presence, in the dog, of factors in plaque capable of attracting neutrophils. This assumption is in agreement with *in vitro* experiments by e.g. Tempel et al. (1970), Miller, Umana & Folke (1971), Lindhe & Helldén (1972) and skin-window tests by Attström & Egelberg (1971). They observed that human dental plaque contained factors chemotactic for neutrophils. The assumption is further supported by data recently reported by Helldén & Lindhe (1973), who showed that a filtrate from newly formed dental plaque, applied topically to the margin of healthy gingiva in dogs, stimulated gingival exudation and enhanced the leukocyte migration, i.e. produced signs of subclinical gingivitis.

Clinical gingivitis and periodontal breakdown

Following 3-4 weeks of undisturbed plaque formation the gingiva in all dogs started to turn red and oedematous. The Gingival Index scores then increased during the following 3-5 months without tooth cleaning, but thereafter remained almost unaltered during a final 12 month period. Although the colour, texture and bleeding tendency of the marginal gingiva remained unaltered, the exudate scores increased significantly during the final 10 months. In view of the observation by Egelberg (1964) and Oliver et al. (1969) of a close relationship between gingival exudation and size and round cell density of the infiltrated gingival connective tissue, the present data on gingival exudation suggest that from the eighth month and throughout the entire longitudinal study there was a gradual deterioration of the gingiva. This assumption is supported by the pocket

depth measurements, which showed a continuous clinical loss of attachment from the sixth month onwards of neglect of oral hygiene.

The observation of a gradual deterioration of the gingiva and periodontal tissues, which cannot be detected from changes in gingival colour or texture, corroborates data presented by Schroeder, Münzel-Pedrazzoli & Page (1973a). They studied the gingival lesion in man by morphometric and biochemical analysis of gingival biopsies and found no correlation between the clinical state of the gingiva, as evaluated by PM-measurements (Mühlemann & Mazor 1958) and the biochemical, volumetric and numerical density parameters used.

During the final 6 months of plaque accumulation a recession of the gingival margin occurred around some molars and premolars. This finding combined with increasing loss of attachment also corroborates the finding by Schroeder et al. (1973a). They showed that fibroblasts residing an infiltrated connective tissue often exhibited pronounced signs of cell degeneration. On the basis of this observation Schroeder et al. (1973a) assumed that the collagen-producing activity of the fibroblasts in an inflammatory gingiva was reduced. This would, in combination with the high rate of collagen turnover of the gingiva (Page 1972), eventually lead to loss of connective tissue substance (Schroeder & Page 1972).

Prevention of periodontal disease

Gingival scores and periodontal measurements of tooth areas subjected to mechanical cleaning, i.e. brushed twice a day for 18 months, did not produce evidence of clinical gingivitis or periodontitis. Histological examinations of tissue specimens representing clinically healthy gingiva at the end of the 18 month experiments revealed a smooth and narrow crevicular (junctional) epithelium, and a gingival connective tissue, a periodontal ligament, and an alveolar bone without inflammatory cell infiltrates (III). These findings clearly demonstrate that it is possible to maintain gingival and periodontal health by meticulous plaque control.

In paper I it was reported that chemical cleaning of teeth by topical application of 2 % chlorhexidine digluconate as a daily spray

to teeth and gingiva during a 42 day period resulted in the disappearance of dental plaque and of well established gingivitis. It was also shown that tissue regeneration following gingivectomy in chlorhexidine-treated wounds corresponded to that reported by Hugoson & Lindhe (1971) on gingival wounds in dogs maintained in a plaque-free state by frequent cleaning of the teeth. The fact that the gingiva recovered a healthy appearance may reflect that the antimicrobial agent was not detrimental to the gingival tissues, but exerted its positive effect on the gingiva by its antibacterial properties. This is in agreement with recent results by Rosling, Hamp & Lindhe (1973), which showed that wound healing after standardised gingivectomy in dogs displayed only minor signs of inflammation when the wounds and teeth were treated with 0.2 % chlorhexidine digluconate twice a day during healing. The finding is also consistent with that in similar experiments in humans (Asboe Jörgensen et al. 1972) and demonstrates the plaque-inhibitory and plaque-resolving effects of chlorhexidine. But the studies referred to illustrate only the short-term plaque-inhibitory effect of the drug.

Paper IV gave data on the long-term effect (12 months) of chlorhexidine on the development of plaque, calculus and gingivitis. The results clearly established that it is possible to maintain a plaque- and gingivitis-free state for almost 6 months by two daily topical applications of 0.2 % chlorhexidine digluconate. The results, however, also showed that chlorhexidine treatment for a period exceeding 6 months did not prevent the development of plaque and gingivitis. In fact, once started the gradual development of plaque and gingivitis in chlorhexidine-treated animals followed the same pattern as in non-treated dogs on a soft diet, but was less severe. This is also in agreement with Schroeder et al. (1973b), who treated young dogs with 2 % chlorhexidine digluconate twice a day for 6 months and reported the presence of microbial plaque on premolars and molars at the end of the treatment period.

In paper V it was demonstrated that the newly formed plaque in chlorhexidine-treated animals contained microorganisms with a reduced sensitivity to the drug. The presence of such microorganisms may be the result of either a selection of resistant mutants amongst the normal plaque organisms or a replacement by naturally less sensitive bacteria, which in the absence of selective pressure, may

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multiply and colonise tooth surfaces. Whatever the mechanism, plaque bacteria subjected to long-term chlorhexidine-treatment may form plaque and induce gingival inflammation in dogs. It should be stressed, however, that the observed microorganisms (identified as Proteus, Klebsiella and Citrobacter) have not been found in human dental plaque (Dwyer & Socransky 1968).

CONCLUSIONS

In the present series of experiments it may be summarized that in the Beagle dog:

a healthy non-inflammatory periodontium (gingiva, periodontal ligament, alveolar bone) can be established and maintained by daily meticulous mechanical cleaning of the teeth;

gingivitis can be induced by allowing microbial colonisation on the gingival third of tooth surfaces;

early gingival inflammation can be registered by gingival exudation and increased migration of crevicular leukocytes, and by demonstration of enhanced lactate dehydrogenase and malate dehydrogenase and ATPase activities in epithelial and endothelial cells within, or in the vicinity of, inflammatory cell infiltrates (subclinical gingivitis);

alterations in colour and texture of the gingiva do not become manifest until 3-4 weeks' continuous plaque formation (clinical gingivitis);

undisturbed plaque formation eventually results in loss of fibre attachment and resorption of the marginal alveolar bone (periodontal breakdown); and

chlorhexidine digluconate applied topically to teeth and gingiva prevents the development of gingivitis only as long as the drug inhibits microbial colonisation in the gingival third of tooth surfaces.

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